

Enzyme-linked immunosorbent assays for study of serological relationships and detection of three luteoviruses

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Enzyme-linked immunosorbent assay (ELISA) systems were used to examine serological relationships and to detect three luteoviruses: a vector-non-specific strain of barley yellow dwarf virus from Illinois (BYDV-PAV-IL), a strain of beet western yellows virus from California (BWYV-CA) and a dwarfing strain of soybean dwarf virus from Japan (SDV-D). Indirect ELISA (IND-ELISA) systems detected distant reciprocal serological relationships among all three viruses. This is the first report of a serological relationship between a vector-non-specific strain of BYDV and any strain of SDV. Double antibody sandwich ELISA (DAS-ELISA) systems detected the purified homologous viruses at concentrations as low as 1.6 ng/ml, but did not detect heterologous viruses as concentrated as 800 ng/ml. In contrast, when DAS-ELISA systems were used for detection of the three viruses in sap extracts from infected plants some weak but significant ($P=0.05$) heterologous reactions occurred. The BYDV-PAV-IL DAS-ELISA system usually detected BWYV-CA and sometimes detected SDV-D; the BWYV-CA DAS-ELISA system never detected BYDV-PAV-IL and rarely detected SDV-D; the SDV-D DAS-ELISA system sometimes detected BYDV-PAV-IL and consistently detected BWYV-CA.

INTRODUCTION

The luteoviruses are an economically important group of plant viruses that are transmitted only by aphids, are phloem-limited and occur in relatively low concentration in infected plants (Rochow & Duffus, 1981). These characteristics have hindered virus purification and thus antiserum production and characterization. Recent methods, including the use of enzymes for extraction, have overcome this problem for some luteoviruses (Takanami & Kubo, 1979; D'Arcy *et al.*, 1983).

Diagnosis of luteovirus infection based on enzyme-linked immunosorbent assay (ELISA) is preferable to insect transmission and visual assessment because of its greater sensitivity, reliability and rapidity (Rochow, 1982; D'Arcy, 1984). The work reported here investigated use of ELISA for detection and differentiation of three luteoviruses: barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV) and soybean dwarf virus (SDV). ELISA systems have been developed for these viruses (Lister & Rochow, 1979; Hewings & D'Arcy, 1984; A.D. Hewings,

unpublished), but their reactions in the heterologous systems have not been examined.

ELISA was also used to see if relationships between the viruses could be quantified. Serological relationships among BYDV, BWYV and SDV have been studied in antiserum adsorption and infectivity neutralization tests (Duffus, 1977; Duffus & Rochow, 1978; Rochow & Duffus, 1978), but have not been examined by ELISA.

MATERIALS AND METHODS

Virus propagation

An Illinois strain of BYDV previously shown to be transmitted by both *Sitobion* (= *Macrosiphum*) *avenae* Fabricius and *Rhopalosiphum padi* L. (BYDV-PAV-IL), a California strain of BWYV obtained from J. E. Duffus (BWYV-CA) and a Japanese dwarfing strain of SDV obtained from T. Tamada (SDV-D) were used in all experiments. BYDV-PAV-IL was maintained in oats (*Avena byzantina* C. Koch cv. Coast Black) and transmitted by *R. padi*. BWYV-CA was main-

tained in turnips (*Brassica rapa* L. cv. White Globe Purple Top) and transmitted by *Myzus persicae* Sulz. SDV-D was maintained in quarantine in soybeans (*Glycine max* (L.) Merr. cv. Wayne) and transmitted by *Aulacorthum solani* Kalt. Viruses were purified as previously described (D'Arcy *et al.*, 1983; Hewings *et al.*, 1986).

Antiserum production

Antisera to BYDV-PAV-IL, BWYV-CA and SDV-D were produced in New Zealand white rabbits as previously described for BWYV-CA (Hewings & D'Arcy, 1984). Totals of 1470 µg, 460 µg and 200 µg of BYDV-PAV-IL, BWYV-CA and SDV-D, respectively, were used for antiserum production. Antisera used for comparative studies were collected 13–14 weeks after the first injection. Homologous titres for all three antisera were greater than 1024. Non-specific titres were 4 for BYDV and BWYV antisera, and 32 for SDV antiserum. Antiserum to SDV-D was cross-absorbed with sap from uninoculated soybeans to reduce non-specific reactions. Immunoglobulins (Igs) were purified as previously described for BWYV-CA (Hewings & D'Arcy, 1984).

Sample preparation

Purified viruses were diluted in 0.1 M sodium phosphate buffer, pH 7.0, using 7.8, 8.1 and 7.3 as the extinction coefficients for BYDV-PAV-IL, BWYV-CA and SDV-D, respectively (Hewings *et al.*, 1986; Hewings & D'Arcy, 1986). Extracts from virus-infected and healthy plants were made by finely chopping 1 g of leaf, adding 3 ml 0.1 M sodium phosphate buffer, pH 7.0, and grinding at high speed for 15–30 s in a Tekmar Tisumizer. The samples were then filtered through a layer of Kimwipe tissue.

Double antibody sandwich ELISA (DAS-ELISA)

Immulon I microtitre plates (Dynatech) were coated with 2 µg/ml Ig in 0.05 M carbonate coating buffer, pH 9.6 (Clark & Adams, 1977) for 2 h at room temperature (*c.* 25°C). Unless otherwise indicated 100 µl were used in all steps and, between each step, plates were rinsed three times for 3 min with phosphate-buffered saline with 0.5 ml/l Tween-20 (PBS-T) (Clark & Adams, 1977). Plates were blocked with 200 µl/well 4% polyvinylpyrrolidone in PBS for 2 h at 37°C. Samples were incubated overnight at 4°C, as was alkaline-phosphatase-Ig conjugate diluted 1:200

for BYDV-PAV-IL and BWYV-CA, and 1:600 for SDV-D. Substrate, 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, was incubated 1 h at room temperature and absorbance of each well at 405 nm was read on an ELISA plate reader (Biotek). Values greater than the mean absorbance plus four standard deviations of three to eight control wells of buffer (in tests with purified viruses) or of extracts from uninfected plants (in tests with infected plant extracts) were considered positive (Voller *et al.*, 1978). Three repetitions of each DAS-ELISA experiment, with each virus-antiserum combination replicated three times, were done.

Indirect ELISA (IND-ELISA)

Plates were coated with purified viruses diluted from 100 to 0.2 ng/ml in carbonate buffer for 2 h at 37°C and blocked as in DAS-ELISA. Ig at 2 µg/ml was incubated overnight at 4°C, as was goat anti-rabbit alkaline phosphatase conjugate (Sigma) diluted 1:1000 in PBS-T. Substrate incubation and determination of positive samples were as described for DAS-ELISA. Preliminary serological differentiation indices were calculated (Van Regenmortel, 1982). Repetitions and replications were as in DAS-ELISA.

RESULTS

DAS-ELISA

DAS-ELISA for each virus detected as little as 1.6 ng/ml of purified homologous virus, but failed to detect heterologous viruses, even at concentrations of 800 ng/ml (Table 1). Extracts from virus-infected plants had strong homologous reactions, but also had some weak, but statistically significant ($P=0.05$), heterologous reactions (Table 2). In three experiments the BYDV-PAV-IL DAS-ELISA detected BWYV-CA in 21 of 22 samples from different plants and detected SDV-D in 8 of 22 samples. The BWYV-CA DAS-ELISA never detected BYDV-PAV-IL (0 of 21) and detected SDV-D in only 3 of 22 samples. The SDV-D DAS-ELISA detected BYDV-PAV-IL in 12 of 21 samples and consistently detected BWYV-CA (21 of 21).

IND-ELISA

Indirect ELISA was approximately four times more sensitive than DAS-ELISA in homologous reactions with all three purified viruses. End-

Table 1. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of purified barley yellow dwarf virus (BYDV-PAV-IL), beet western yellows virus (BWYV-CA) and soybean dwarf virus (SDV-D)

Coating and conjugated antibodies	Antigen concentration (ng/ml)	A_{405} with antigen		
		BYDV	BWYV	SDV
BYDV-PAV-IL ^a	800.0	> 1.999	0.103	0.105
	400.0	1.601	0.099	0.100
	200.0	1.025	0.097	0.108
	100.0	0.730	0.098	0.104
	12.5	0.297	0.104	0.103
	6.3	0.153	0.096	0.099
	3.2	0.138	0.104	0.102
	1.6	0.125	0.105	0.108
BWYV-CA ^b	800.0	0.116	1.004	0.117
	400.0	0.112	0.763	0.114
	200.0	0.118	0.622	0.116
	100.0	0.110	0.394	0.110
	12.5	0.116	0.161	0.111
	6.3	0.118	0.137	0.114
	3.2	0.116	0.129	0.116
	1.6	0.121	0.127	0.116
SDV-D ^c	800.0	0.128	0.122	> 1.999
	400.0	0.122	0.117	> 1.999
	200.0	0.120	0.118	> 1.999
	100.0	0.126	0.118	1.613
	12.5	0.117	0.117	0.318
	6.3	0.117	0.116	0.230
	3.2	0.118	0.118	0.175
	1.6	0.121	0.118	0.141

^a Eight control wells, \bar{x} = 0.104, σ = 0.003, +^d = 0.117.

^b Eight control wells, \bar{x} = 0.117, σ = 0.002, + = 0.126.

^c Eight control wells, \bar{x} = 0.125, σ = 0.003, + = 0.138.

^d +, lowest A_{405} value that equals mean (\bar{x}) for uninfected samples plus four standard deviations (σ).

points were in the range 0.2–0.8 ng/ml virus in three experiments. Reciprocal relationships were detected among all three viruses at concentrations of approximately 100 ng/ml or less (Table 3). Calculations of serological differentiation indices (SDIs) from three experiments gave values of 6–8 for BYDV-PAV-IL and BWYV-CA, 6–8 for BYDV-PAV-IL and SDV-D and 8–>9 for BWYV-CA and SDV-D. Thus, relationships among all three viruses are distant (SDI > 4) (Van Regenmortel, 1982).

DISCUSSION

Serological relationships among luteoviruses

have been studied using at least seven different serological tests and the results indicate that relationships are common (Rochow & Duffus, 1981). Duffus (1977) examined relationships among three strains of BYDV (MAV, PAV, RPV), four of BWYV (1, 3, 7, E) and two of SDV (DS, Y) by antiserum adsorption and infectivity neutralization. Using the same methods, Duffus & Rochow (1978) studied BYDV and BWYV relationships. These authors reported positive reactions between BWYV and antisera to BYDV-PAV and to SDV-D. However, no reaction was found between BYDV-PAV and BWYV antiserum, and purified SDV was unavailable for tests. Our results with indirect ELISA indicate

Table 2. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of extracts from plants infected with barley yellow dwarf virus (BYDV-PAV-IL), beet western yellows virus (BWYV-CA) and soybean dwarf virus (SDV-D) or uninoculated plants as controls

Coating and conjugated antibodies	Plant ^a	Virus	<i>A</i> ₄₀₅ with extract	
			Infected	Uninfected
BYDV-PAV-IL	Oats	BYDV	> 1.999 + ^b	0.193
			> 1.999 +	0.197
			> 1.999 +	0.237
	Turnips	BWYV	0.139 +	0.085
			0.197 +	0.086
			0.215 +	0.080
	Soybeans	SDV	0.113	0.115
			0.126	0.004
			0.147 +	0.002
BWYV-CA	Oats	BYDV	0.087	0.108
			0.098	0.117
			0.085	0.096
	Turnips	BWYV	0.403 +	0.064
			0.457 +	0.073
			0.438 +	0.062
	Soybeans	SDV	0.083	0.063
			0.076	0.061
			0.079	0.083
SDV-D	Oats	BYDV	0.129 +	0.082
			0.181 +	0.097
			0.086	0.071
	Turnips	BWYV	0.172 +	0.083
			0.311 +	0.083
			0.167 +	0.072
	Soybeans	SDV	0.732 +	0.246
			0.747 +	0.249
			1.117 +	0.279

^a Extracts prepared by homogenizing 1 g uninfected tissue, or tissue infected with the virus indicated, in 3 ml 0.1 M phosphate buffer, pH 7.0. Each extract was tested in all three DAS-ELISA systems.

^b *A*₄₀₅ value \geq mean of uninfected samples plus four standard deviations.

that all three viruses are related, with positive results in all reciprocal tests. This is the first report of a serological relationship between a vector-non-specific strain of BYDV (BYDV-PAV) and any SDV strain. Indirect ELISA is a simple, sensitive method to detect such distant (SDI > 4) relationships.

The SDI values that we have calculated must be considered preliminary, as only one bleed from

one rabbit injected with each virus was used in our tests. In future studies more bleeds from more animals will allow the calculation of more accurate SDI values between different luteoviruses and strains of a single virus.

Currently there are 14 members and 19 probable or possible members of the luteovirus group (Matthews, 1982). There is, however, very little quantitative information on serological related-

Table 3. Concentrations of three purified luteoviruses detected by indirect enzyme-linked immunosorbent assay (IND-ELISA) in homologous and heterologous reactions

Antibodies	Detectable virus concentration with coating antigen ^a		
	BYDV-PAV-IL	BWYV-CA	SDV-D
BYDV-PAV-IL	0.4	25.0	100.0
BWYV-CA	25.0	< 0.2	100.0
SDV-D	50.0	> 100.0	0.8

^a Lowest virus concentration (ng/ml) with $A_{405} \geq$ mean of eight buffer controls plus four standard deviations.

ness among these viruses. Indirect ELISA has the potential to provide such information and to help clarify the chaotic state of luteovirus taxonomy.

Detection of luteoviruses by DAS-ELISA is certainly feasible but problems of identification, due to heterologous reactions, may occur. With the three viruses studied here such heterologous reactions were weak, so correct diagnosis could be made. As additional strains and viruses are included in such a detection system, the interpretation becomes more complex.

The difference in reactions of the three luteoviruses in DAS-ELISA when in plant extracts or as purified virus is noteworthy. No heterologous reactions were seen with purified virus preparations at concentrations greater than any likely to be found in extracts from infected plants. In contrast, significant heterologous reactions were found when extracts from infected plants were tested. These differences may reflect differences in the condition of the viruses in plant extracts or when purified and suspended in buffer. Purification procedures select for intact virions and we used or froze such preparations immediately. In contrast, plant extracts contain intact virions and virus components, including capsid subunits. Prolonged exposure to such extracts, as during the overnight sample incubation in DAS-ELISA, may also cause proteolytic degradation of virions (Van Regenmortel, 1982).

When preparations of purified intact virions are injected into rabbits for antiserum production, the virions may degrade into constituent subunits and antibodies specific to these breakdown products may be produced (Van Regenmortel, 1982). In our system these antibodies would then react with serologically related subunits in extracts from infected plants. That not

only homologous but also heterologous reactions were seen in such extracts, while no heterologous reactions were seen with purified intact virions, indicates that the dissociated protein capsids of the three luteoviruses in this study may be serologically more closely related than are the intact viruses. This has been reported previously for bromoviruses (Rybicki & Von Wechmar, 1981), potyviruses (Shepard *et al.*, 1974) and tobamoviruses (Van Regenmortel & Burckard, 1980).

Support for this hypothesis is that purified BWYV-CA virions are extremely difficult to disrupt *in vitro* (A.D. Hewings & C.J. D'Arcy, unpublished) and antiserum to this virus had the fewest and weakest cross reactions in this study. Therefore, BWYV-CA may also be extremely stable in the rabbit and few cross-reactive antibodies to subunits are produced.

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